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Award Number: DAMD17-03-1-0742

TITLE: Identification of the Molecular Determinants of Breast Epithelial Cell Polarity

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REPORT DATE: October 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2004	Annual (8 Sep 2003 - 7 Sep 2004)

4. TITLE AND SUBTITLE
Identification of the Molecular Determinants of Breast Epithelial Cell Polarity

5. FUNDING NUMBERS
DAMD17-03-1-0742

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Cell polarity is a fundamental property of tissue architecture and loss of polarity has been linked to progression of malignancy in cancer. Both cell-cell and cell-extracellular matrix interactions are involved in the establishment and maintenance of cell polarity. However, molecular information on how these interactions and polarity are coordinated and how the integrity of these interactions and polarity are modified in cancer is still limited.

Although the Ras family proteins control a wide variety of cellular processes, the function of Rap1, which is most closely related to Ras, remains unclear. The original characterization of Rap1 in fibroblasts indicated Rap1 antagonizes Ras signal by competing effectors. However, later studies revealed that like Ras, Rap1 can stimulate MAPK pathway in several cell types. In addition, recent reports showed that Rap1 stimulates integrin-dependent adhesion of T-cells and regulates the distribution of adherens junctions in *Drosophila* epithelial cells.

I hypothesized that Rap1 could be a potential molecule which coordinates, cell-cell, cell-ECM interactions, and cell polarity in breast epithelial cells. The analyses of malignant breast epithelial cells transfected with dominant-active and dominant-negative form of Rap1 in a three-dimensional culture model indicate that Rap1 is involved in the regulation of malignant phenotype of breast epithelial cells.

14. SUBJECT TERMS
No subject terms provided.

15. NUMBER OF PAGES
24

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Loss of tissue polarity and increased proliferation are the characteristic alterations of the breast tumor phenotype. Whereas epithelial-mesenchymal transitions occur late during metastatic progression in tumorigenesis, loss of epithelial polarity is thought to be early event in cancer[1]. A number of studies indicate that cell-cell and cell-extracellular matrix (ECM) interactions play an essential role in the establishment and maintenance of cell polarity[2]. Cell-cell and cell-ECM interactions have been suggested to cross-talk with each other, and this coordinated cross-talk is crucial for regulating homeostasis and tissue architecture. However, the molecular mechanisms that couple cell-cell and cell-ECM interactions, and pathways that integrate this context into the establishment of cell polarity and tissue architecture remain largely unresolved to date.

Dr. Bissell's laboratory has developed a three-dimensional (3D) culture system with laminin-rich basement membrane (lrBM) in which malignant human breast cells can be reverted to a normal phenotype by exposure to inhibitors of EGF Receptor (EGFR) or β1-integrin[3]. This *ex vivo* assay system provides physiologically relevant models to address those unresolved questions.

Currently, I am using this assay system to study the regulators of tight junctions by analyzing the role of a small G protein, Rap1, which is potentially involved in cell polarity and cell growth through the regulation of both cell-cell and cell-ECM interactions.

BODY (All figures are in Appendix I)

A requirement of cell polarity in epithelia is tight junctions (TJs), which are the most apical components of the cell-cell junctional complexes. TJs function as the "fence" separating apical from basolateral membrane domains, and thus play important roles in the regulation of cell polarity. Recent studies show that TJs are comprised of three types of integral membrane proteins; occludin, claudins, and JAM. Various cytoskeletal and signaling molecules are recruited at their cytoplasmic surface to form macromolecular complexes which are responsible for the regulation of cell polarity.

I first examined the expression levels of TJ components in non-malignant (S1) and malignant (T4) cells in 2D and 3D lrBM culture conditions (Fig. 1; Task 1). The expression of occludin is higher in T4 cells as compared to S1 cells, and is down-regulated when grown in 3D lrBM in both types of cells. Claudin has a multi-protein family. Only caludin-1 is detectably expressed both in S1 and T4 cells. In 2D, the claudin-1 appears to be equally expressed in S1 and T4 cells, while in 3D lrBM T4 cells have higher expression than S1 cells. JAM, the immunoglobulin superfamily molecule, is expressed at lower levels in 2D cultured T4 cells only. Several PDZ proteins, which have been implicated in regulating membrane protein localization, are concentrated at TJs. ZO-1, ZO-2, and MUPP1 appear to be expressed higher in 3D lrBM than in 2D, although there are not significant differences in their expression levels between in S1 and T4 cells. PAR-3, another PDZ protein which is involved in cell polarity not only in epithelial cells but also in neuronal cells, shows comparable expression in all conditions. Atypical PKC

(aPKC) forms complex with PAR-3 and its kinase activity is required for the asymmetric differentiation of the premature junctional complexes during epithelial cell polarization. $G_{\alpha 12/13}$, one of the heterotrimeric G-protein, binds to ZO-1 and activated $G_{\alpha 12/13}$ increases paracellular permeability of epithelial cells. The expression pattern of both singling molecules is similar; higher in 3D lrBM than in 2D and same between S1 and T4 cells. In summary, three integral membrane proteins exhibit different expression patterns, but both occludin and claudin-1 are expressed higher in malignant T4 cells as compared to S1 cells in 3D lrBM culture conditions. However, most of cytoplasmic proteins have similar characteristics in their expression although the pattern does not correspond with any of integral membrane proteins.

To extend the knowledge of the expression of TJ components and the progression towards malignancy, several non-malignant, malignant (non-invasive), and invasive breast epithelial cells were analyzed (Fig. 2). Interestingly, claudin-1, -3, and -4 are undetectable in all of three invasive breast cells. In particular, the expression pattern of claudin-3 is quite unique; as its expression is detected only in non-invasive malignant cells and not in non-malignant or invasive cells all examined. For the cytoplasmic proteins, there are only slight differences among these breast epithelial cell lines.

Since the characterization of ultrastructure of cells could help to connect cellular phenotypes with mechanisms regulating polarity, electron microscopic studies of 2D and 3D lrBM cultured S1 and T4 cells were performed (Fig. 3A and 3B; Task 2). In 2D culture, both S1 and T4 cells have microvilli at apical surfaces and cell-cell junctional complexes at the most apical part of lateral membranes, indicating that apico-basal polarity is established even in malignant T4 cells. In 3D lrBM culture, desmosomes and hemidesmosomes are highly visible in lateral and basal domains of S1 cells, respectively. On the other hand, desmosomes and hemidesmosomes are intermingled at lateral domains in T4 cells suggesting that basal polarity is impaired. The addition of EGFR inhibitor tyrophostin AG1478 to T4 cells restored distinct localizations of desmosomes and hemidesmosomes and thus established basal polarity. TJs are difficult to identify in Fig. 3B as they are tiny structures below the resolution of current EM.

From the data described in Fig. 1 and Fig. 2, the alterations of TJ components associated with phenotypic difference between S1 and T4 cells appear to be a consequence rather than cause of deregulated polarity so far. Therefore, instead of proceeding to examine gain and loss of function analyses of TJ components (Task 3), it seemed to be appropriate shifting the focus slightly to identify novel molecular mechanisms or pathways that regulate TJ proteins as well as polarity in normal and tumorgenic cells.

The Ras superfamily of GTPases controls a wide variety of cellular processes. Rap1 is a member of the subset of GTPases that are most closely related to Ras itself. Whereas the biological function of Ras, particularly its role in cellular growth and differentiation, are well established, the function of Rap1 is poorly understood. Since Rap1 was originally identified as a suppressor of Ras-mediated oncogenic transformation in fibroblasts, Rap1 was thought to compete for Ras effectors[4]. However, later studies in neuronal cells revealed that Rap1 can stimulate MAPK through B-Raf and that overexpression of Rap1

was able to induce oncogenic transformation[5]. These contradictory results suggest that the effect of Rap1 signal is cell-type dependent. Recent studies indicate that Rap1 could be involved in the regulation of both cell-cell and cell-ECM interactions as well as cell proliferation. Rap1 stimulates integrin-dependent adhesion of human T cells, and adhesion of T cells can be blocked by expression of dominant-negative form of Rap1[6]. In *Drosophila*, the distribution of adherens junctions in epithelium is controlled by an orthologue of Rap1[7].
Based on these reports and our previous data, I hypothesized that Rap1 could be a potential molecule which coordinates cell-cell interactions, cell-ECM interactions, and cell polarity in breast epithelial cells.

I determined the expression and activity of Rap1 in non-malignant (S1) and malignant (T4) cells (Fig. 4) in 2D and 3D lrBM culture conditions. Both S1 and T4 cells expressed almost same amount of Rap1 however, the activity of Rap1 is higher in T4 cells than in S1 cells cultured in 3D lrBM, suggesting that Rap1 activity might be related to malignant phenotype of breast epithelial cells.

To investigate if the activation of Rap1 is functionally involved in the progression of malignancy in breast epithelial cells, I introduced YFP-tagged dominant-negative form of Rap1 in T4 cells, and obtained stable clones (T4-Rap1DN). The morphology of T4-Rap1DN cells did not show significant difference from that of control cells expressing YFP (T4-YFP) in 2D culture (Fig. 5A). However, a remarkable effect was observed when these cells were cultured in 3D lrBM (Fig. 5B). T4-YFP cells behaved in a same manner with parental T4 cells; continuing to proliferate and forming large and disorganized colonies similar to structures formed by primary tumor cells. On the other hand, T4-Rap1DN cells underwent morphogenesis similar to the non-malignant breast epithelial cells and formed organized acinus-like structures.

In well-polarized epithelial cells, α6-integrin is localized at basal domains, whereas GM130, which is a component of Golgi apparatus, is distributed at the apical side of nucleus. To determine whether polarity is established in these T4 transfectants in 3D lrBM culture, localizations of α6 integrin and GM130 were examined (Fig. 6). In T4-YFP cells, α6-integrin was found in entire cell membrane and GM130 was randomly distributed, indicating that polarity was not established. In contrast, basal localization of α6- integrin and organized localization of GM130 above nucleus were observed in T4-Rap1DN cells by immunofluorescence. Therefore, the reduction of Rap1 activity was able to restore normal polarity in malignant T4 cells.

Next, I established T4 cells transfected with dominant-active form of Rap1 (T4-Rap1DA) to determine whether the increased Rap1 activity could drive T4 cells into more malignant phenotype. The morphology of T4-Rap1DA cells looked similar to T4-YFP cells in 2D as well as in 3D lrBM culture (Fig. 7A, B). In addition, there was not a difference in colony size in 3D rlBM culture between two cell lines.
Our previous studies demonstrated that EGFR inhibitor AG1478 strongly suppressed proliferation of T4 cells and that tissue polarity is restored in 3D lrBM culture in the presence of AG1478[8]. I examined the effects inhibiting EGFR signal in T4-YFP and T4-

Rap1DA cells by treatment cells with 50nM AG1478. Although T4-YFP cells were reverted to non-malignant phenotype; the growth was suppressed and α6-integrin was specifically localized at basal domains (Fig. 8A), the T4-Rap1DA cells continued to proliferate, formed large aggregated colonies, and had non-polarized distribution of α6-integrin (Fig. 8B).

To quantify cell proliferation of T4-YFP, T4-Rap1DA, and T4-Rap1DN cells in 3D lrBM culture, I measured cell number in a single spheroid at day3, day5, and day8.
As shown in Figure 9, T4-YFP and T4-Rap1DA cells grow at a similar rate. In the presence of 50nM AG1478, the growth of T4-YFP cells was 80% decreased. However, T4-Rap1DA cells continue to proliferate at the almost same rate with and without AG1478. In the case of T4-Rap1DN cells, cell number per spheroid was less than 30% that of T4-YFP cells, but 1.5~2 fold more than that of T4-YFP cells treated with AG1478.

When normal mammary epithelial cells are cultured in 3D rlBM, they proceed through morphogenetic steps to achieve a hollow glandular architecture. During the early steps of morphogenesis (~day3), apicobasal polarity becomes evident within cell clusters. It is prior to start the lumen formation. Around day6, cells in direct contact with lrBM become well-polarized, whereas cells lacking contact with rlBM are poorly polarized. The cells which are not in contact with the lrBM begin to die by apoptosis about day8. To determine whether T4-YFP cells reverted with AG1478 and T4-Rap1DN cells follow a similar morphogenesis program to the normal mammary epithelial cells, I cultured both cell lines in 3D rlBM for 15 days. T4-YFP cells showed growth arrest and retained apicobasal polarity however, less than 2% spheroids had formed a lumen at day 15 (Fig. 10A). On the other hand, lumen formation was observed in more than 60% spheroids derived from T4-Rap1DN cells without AG1478 (Fig. 10B). This morphological difference suggests that molecular mechanisms of the reversion of malignant phenotype are different from the inhibiting EGFR signal by AG1478 and inhibiting Rap1 signal.

Another aspect of the advanced tumor phenotype is increased invasiveness. To determine if Rap1 signaling affects the invasiveness of T4 cells, migration and invasion assay were performed using a transwell filter assay. As compared to T4-YFP cells, both T4-Rap1DA and T4-Rap1DN cells showed increased motility although much a higher increase was detected in T4-Rap1DA cells than with the T4-Rap1DN cells (Fig. 11A). In the invasion assay, T4-Rap1DA cells invaded four-fold more than T4-YFP cells did however, only a small number of T4-Rap1DN cells invaded (Fig. 11B). Therefore, activation of Rap1 appears to promote several aspects of malignancy in breast epithelial cells i.e. intervers with polarity, and increases invasion and migration.

To dissect out the molecular mediators of these cellular phenotypes in malignant breast cells as a result of Rap1 signal modulation, I analyzed EGFR-MAPK pathways (Fig. 12). By western analysis, total EGFR and MAPK (Erk1/2) expression levels did not change in either T4-Rap1DA or T4-Rap1DN cells as compared to T4-YFP cells. The activities of EGFR and MAPK were determined by phospho-specific antibodies. No obvious reduction of phospho-MAPK or p90RSK was detected in T4-Rap1DN cells; however, this data might mean that the basal levels of the phosphorlyation of these molecules are

not being modulated. In T4-Rap1DA cells, phospho-MAPK level was up-regulated, whereas phospho-EGFR appears to be unchanged as compared to T4-YFP cells. p90RSK which is one of the downstream targets of MAPK, was also activated in T4-Rap1DA cells which may indicate that activation of Rap1 in T4 cells can stimulate pathways downstream of MAPK.

In our previous studies, inhibition of EGFR signal with AG1478 affected the expression of β1-integrin and likewise, treatment of T4 cells with β1-integrin inhibitory antibody reduced expression of EGFR in 3D rlBM. Since Rap1 is thought to modulate integrin signaling, I analyzed β1-integrin and FAK in Rap1 transfeced cells (Fig. 13). In T4-Rap1DA cells, total β1-integrin and FAK levels were almost same and phospho-FAK was slightly increased compared to T4-YFP control transfected cells, suggesting that the β1-integrin pathway as well as the MAPK pathway might be stimulated by Rap1 in T4 cells. However, even though T4-Rap1DN cells exhibited non-malignant phenotype, the expression of β1-integrin in addition to EGFR were not reduced as is seen in the reverted AG1478 treated T4 cells.
Therefore, molecular mechanisms of reversion of malignant phenotype of T4 cells by the reduction of Rap1 activity are not identical to EGFR inhibition by AG1478. These biochemical data along with morphological difference between T4-Rap1DN cells and T4 cells reverted with AG1478 may indicate that the effect of Rap1 does not modulate EGFR or β1-integrin pathways in the same manner. Furthermore, since lumen formation is observed only in T4-Rap1DN cells, the degree of apical polarity establishment might be advanced in T4-Rap1DN cells as compared to T4 cells reverted with AG1478. This data could suggest that Rap1 affects expression or localization of TJ proteins and thus modulates TJ formation as well as cell polarity. Further analyses of T4-Rap1DN as well as T4-Rap1DA cells would lead to understand the relationship between Rap1 signaling and TJs, and to delineate how TJ regulation plays an important role in differentiation and deregulation would result in tumorigenesis.

FUTURE WORK

- Determine activity of β1-integrin in Rap1 transfectants using antibodies specifically recognizing active forms of β1-integrin.
- Determine expression and localization of TJ proteins in T4-Rap1DN cells.
- Determine tumorigenic and metastatic potential of Rap1 transfectants *in vivo* by injections of cells into subcutaneous and tail vein of nude mice.
- Determine Rap1 activity in normal and malignant breast tissues.
- Establish normal mammary epithelial cells expressing dominant-active Rap1 to examine if the activation of Rap1 is sufficient to induce malignant phenotype and/or deregulated polarity in normal cells.

Key Research Accomplishments

- Among tight junction components, occludin and claudin-1 differentially expressed between 3D lrBM cultured S1 and T4 cells.
- Claudin-3 is specifically expressed in non-invasive malignant breast epithelial cells and not in non-malignant or invasive cells.
- Ras superfamily small G protein Rap1 is activated in malignant T4 cells than in non-malignant S1 cells.
- Malignant T4 cells expressing dominant-negative Rap1 restores growth arrest, polarity, and organized structure similar to the non-malignant cells cultured in 3D lrBM.
- Additional activation of Rap1 in malignant T4 cells provides resistance to growth suppression by EGFR inhibitor AG1478.
- The invasiveness of malignant T4 cells is enhanced by activation of Rap1.
- Dominant-active Rap1 increases phosphorylation levels of MAPK and downstream target p90RSK in malignant T4 cells.

Reportable Outcomes

Itoh M. and Bissell M.J. (2003). The organization of tight junctions in epithelia: implications for mammary gland biology and breast tumorigenesis. Journal of Mammary Gland Biology and Neoplasia. 8(4):449-462.

Conclusions

For the study of normal human biology and associated disease progression, the development of physiologically relevant experimental models is crucial. Even though animal models such as transgenic and knock out mouse can be quite informative, cell-based models facilitate systematic analyses that address, at the molecular level, how normal organ structure and function are maintained or how the balance is lost in cancer. A 3D lrBM culture system using the HMT-3522 human breast tumor progression series developed by Dr. Bissell's laboratory has been utilized as a one of the assay models for this purpose. The reversion assay of malignant T4 cells into non-malignant phenotypes with inhibitors against EGFR or β1-integrin provides good example of how the regulation of signaling pathways in 2D and 3D differs and how the 3D assay has several advantages as a physiologically relevant system.

The goal of this project is to identify molecules and pathways involved in a tumor's escape from growth arrest in which they loose epithelial polarity and form disorganized structures, especially focusing on the regulation of TJs. To address this question, I am

currently taking a candidate molecule approach and analyzing a small G protein Rap1. Using 3D lrBM assay system, I proved that activity of Rap1 is involved in the malignant phenotype of T4 cells. The reduction of Rap1 activity in T4 cells by introducing dominant-negative form of Rap1 can revert the malignant phenotype in 3D lrBM; cell growth is arrested, polarity is established, and furthermore a lumen is formed. These features are very similar to the morphogenetic process of normal mammary epithelial cells. On the other hand, the additional activation of Rap1 drives T4 cells increased the malignancy of these cells in several aspects. First, T4 cells overexpressing dominant-active form of Rap1 escape from growth suppression by treatment with 50nM AG1478 which inhibits EGFR signal, whereas growth of T4 cells is greatly suppressed. Second, cell invasiveness which could reflect metastatic ability of cells *in vivo* is increased. Taken together, modulation of Rap1 activity could influence cell growth and polarity in malignant breast epithelial cells in physiologically relevant culture system. The question is what molecular mediators are involved in these processes. The activated MAPK pathway in dominant-active Rap1 expressing T4 cells may be one of the contributing factors. Further biochemical analyses will provide detailed understanding how Rap1 signal affects TJs, cell polarity, and tissue architecture in breast epithelial cells.

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245.

Appendix I

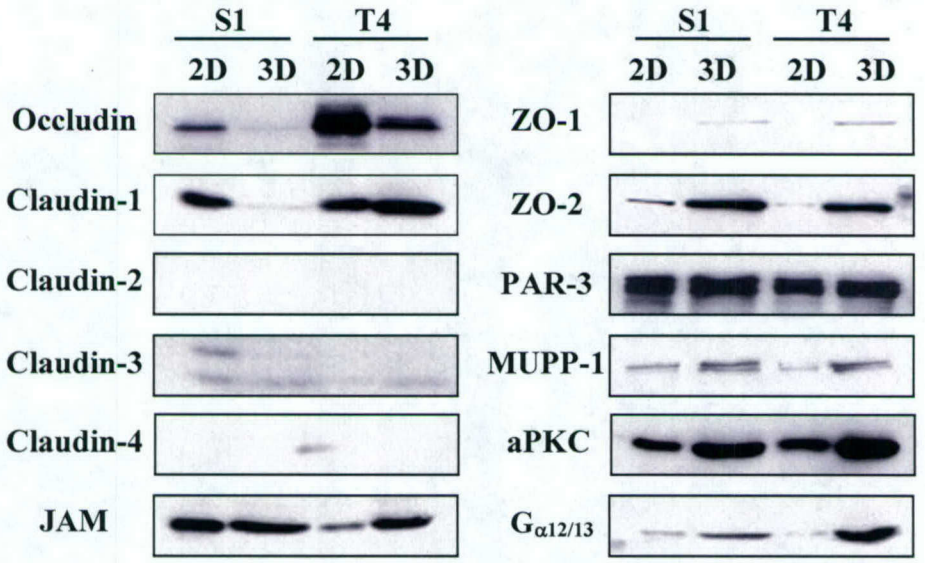


Figure 1 Expression of tight junction components in HMT-3522 human mammary epithelial cells cultured in 2D and 3D lrBM.

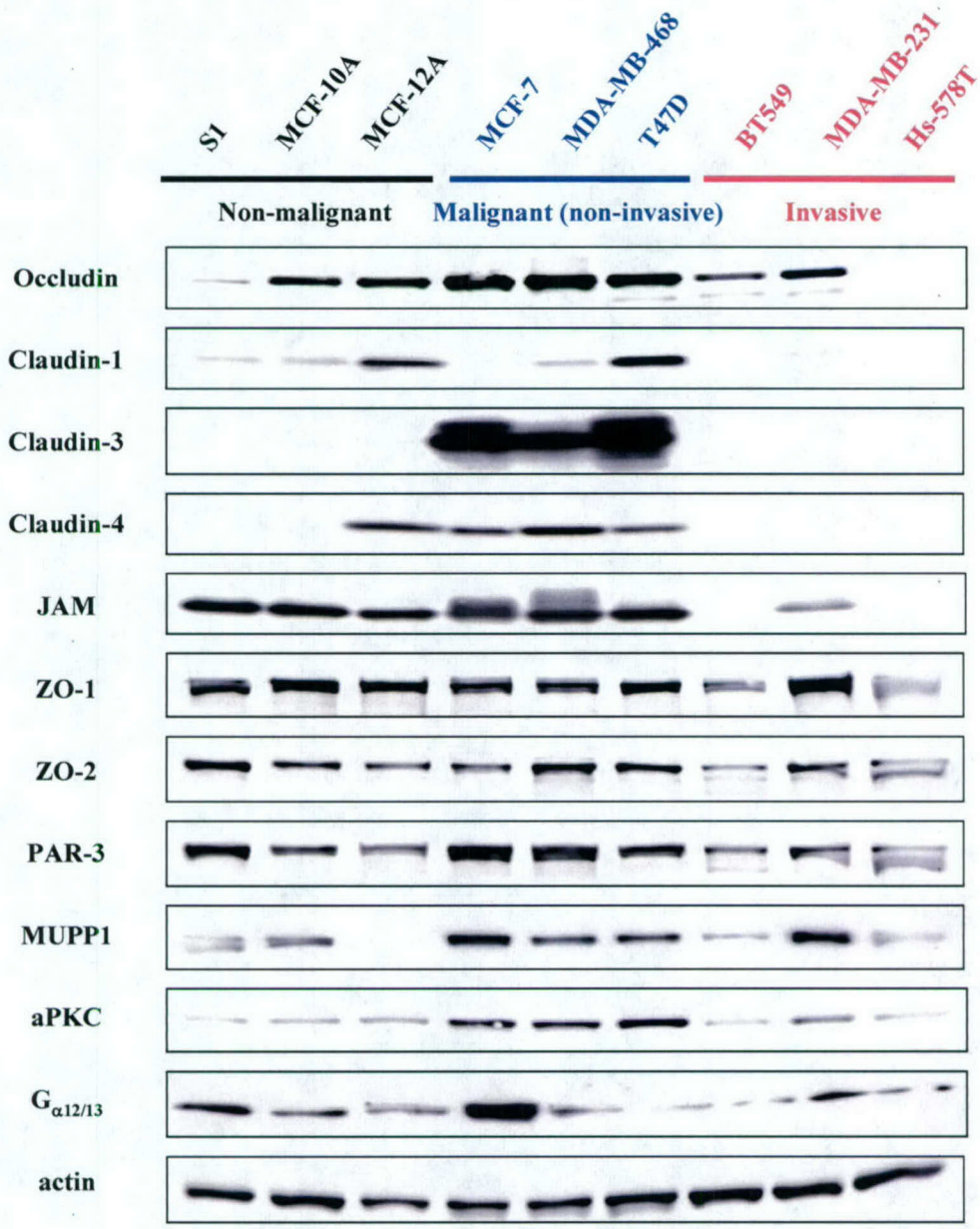


Figure 2 Expression of tight junction components in non-malignant, malignant (non-invasive), and invasive human mammary epithelial cells.

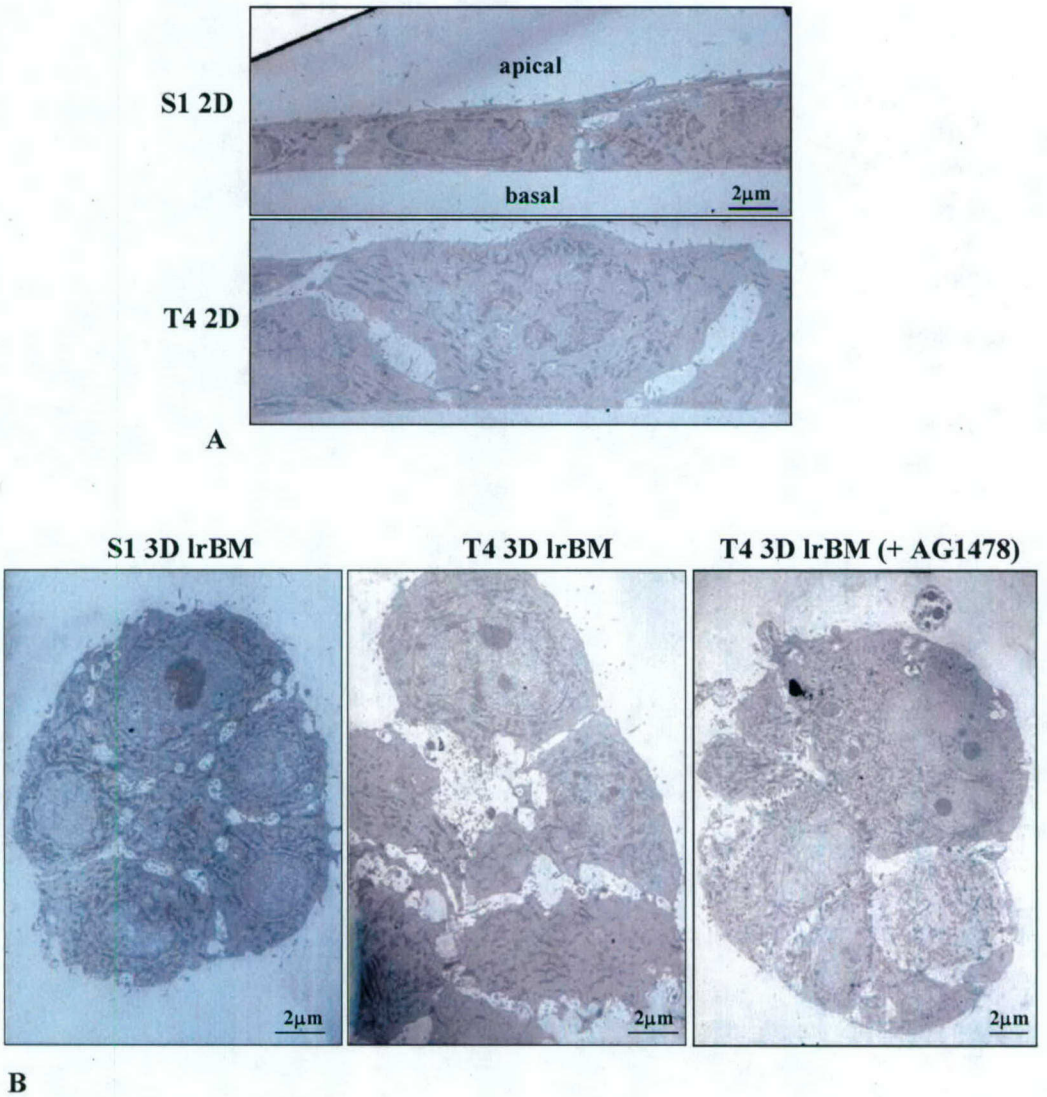
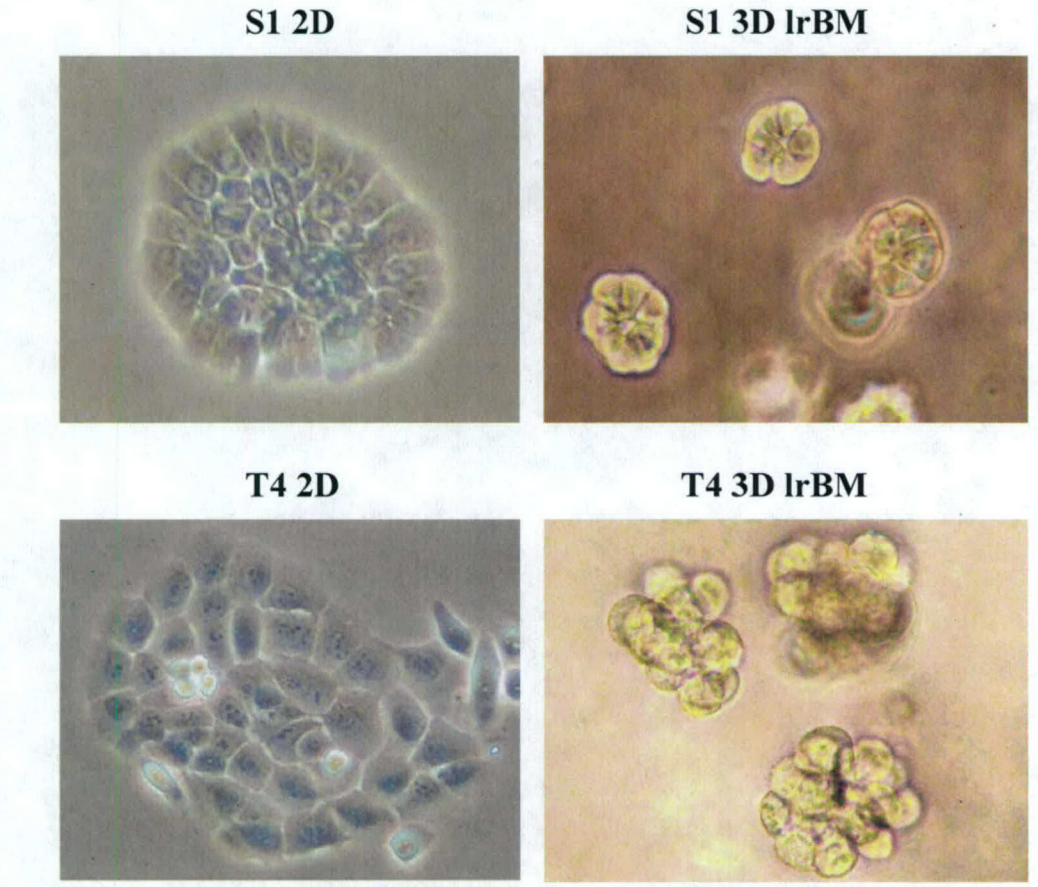
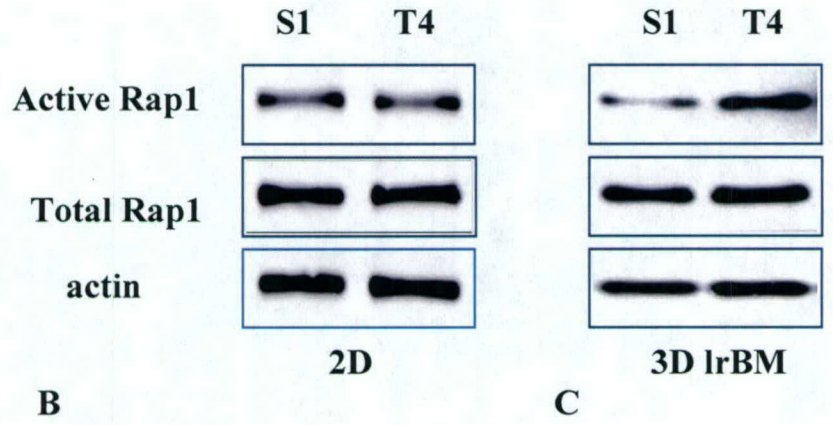


Figure 3 Electron microscopic images of S1 and T4 cells cultured in 2D (A) and in 3D lrBM (B).



A



B C

Figure 4 **A** Morphology of S1 and T4 cells in 2D and 3D lrBM culture. **B, C** Expression and activity of Rap1 in S1 and T4 cells in 2D (**B**) and 3D lrBM (**C**).

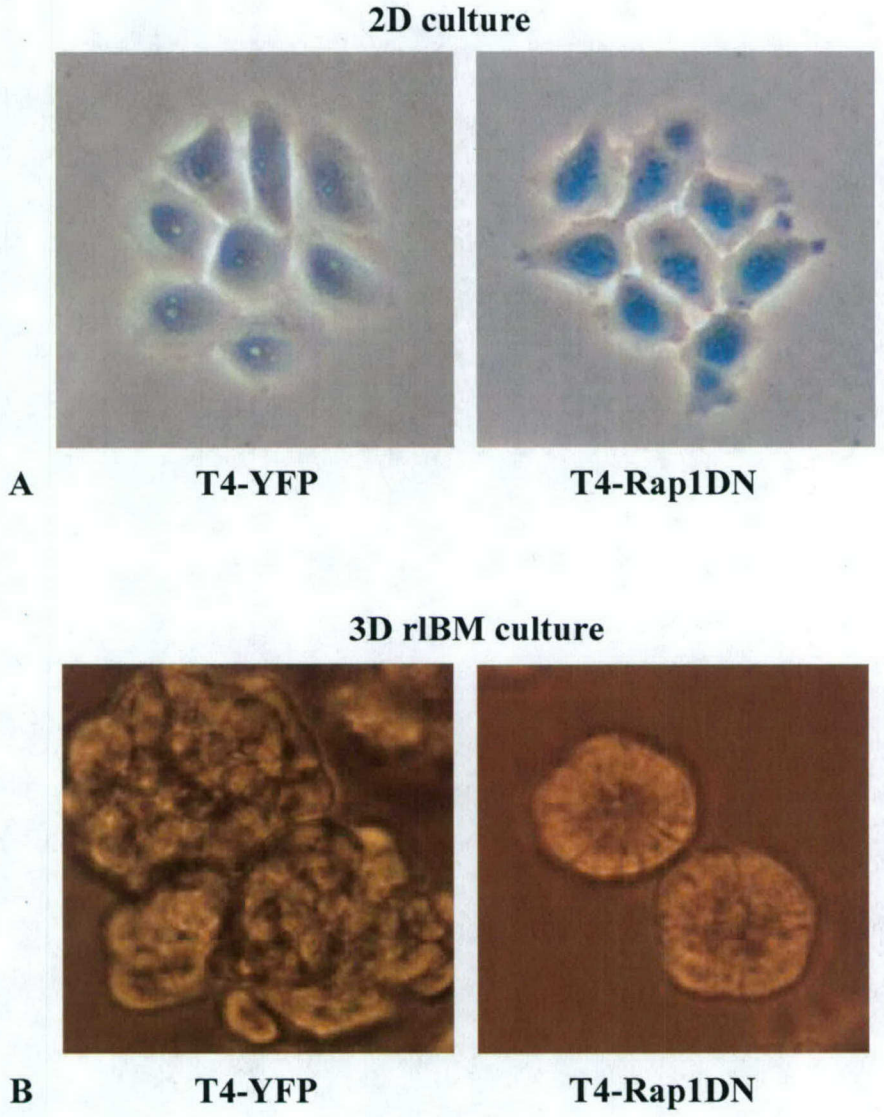


Figure 5 Morphology of T4-YFP and T4-Rap1DN cells cultured in 2D (**A**) and 3D lrBM (**B**)

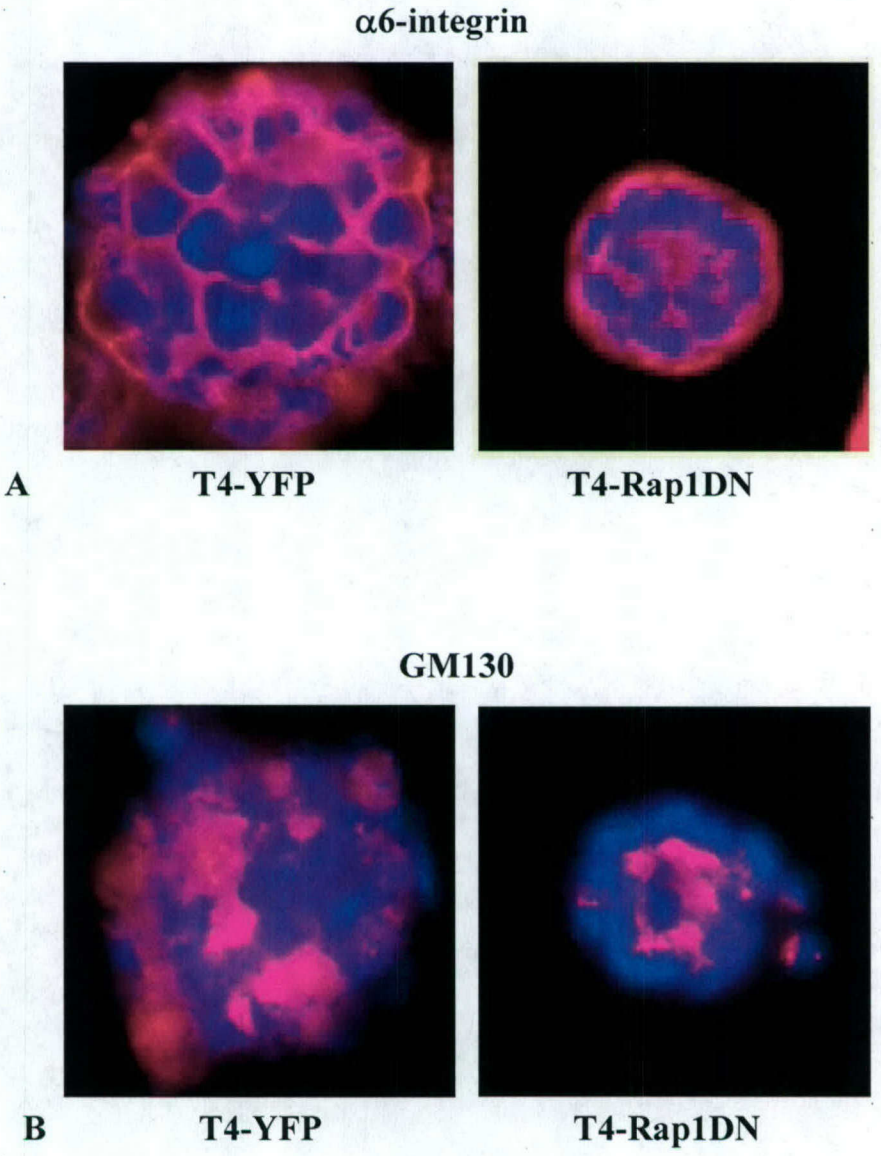


Figure 6 Localization of α6-integrin (**A**) and GM130 (**B**) in T4-YFP and T4-Rap1DN cells cultured in 3D lrBM.

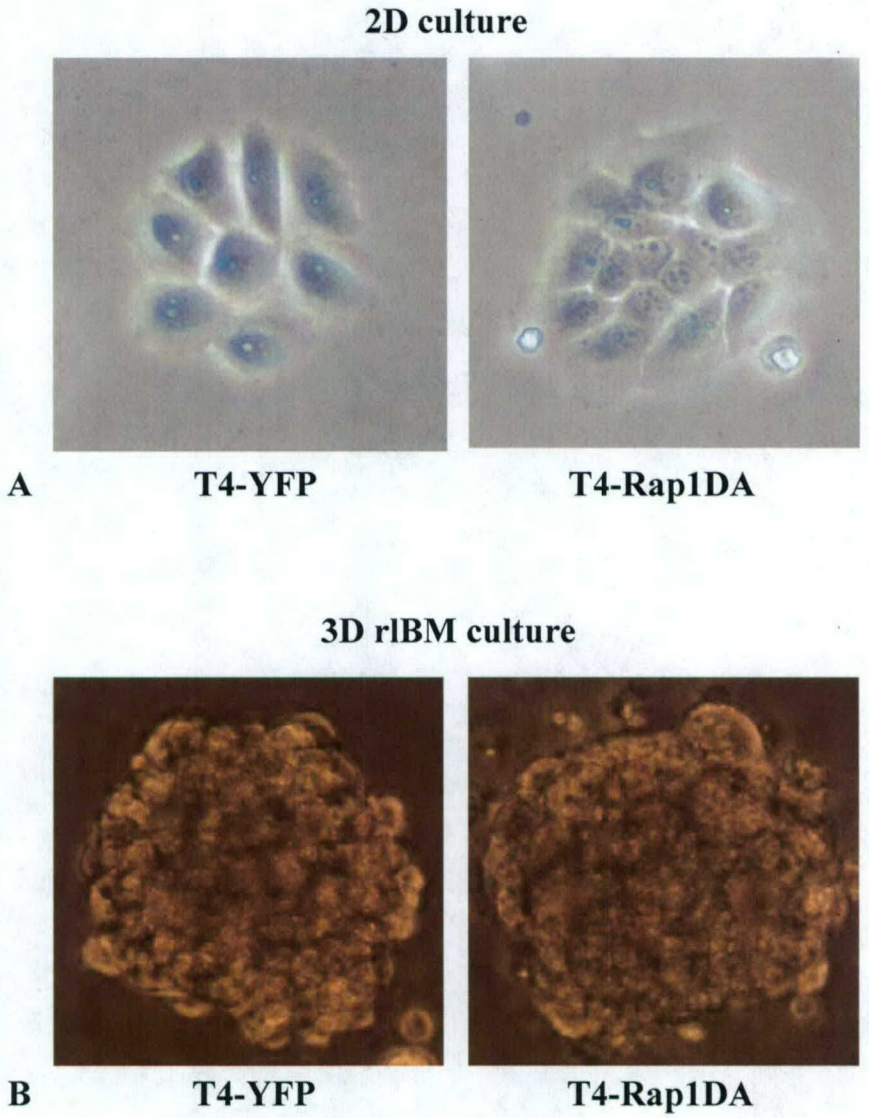


Figure 7 Morphology of T4-YFP and T4-Rap1DA cells cultured in 2D (**A**) and 3D lrBM (**B**).

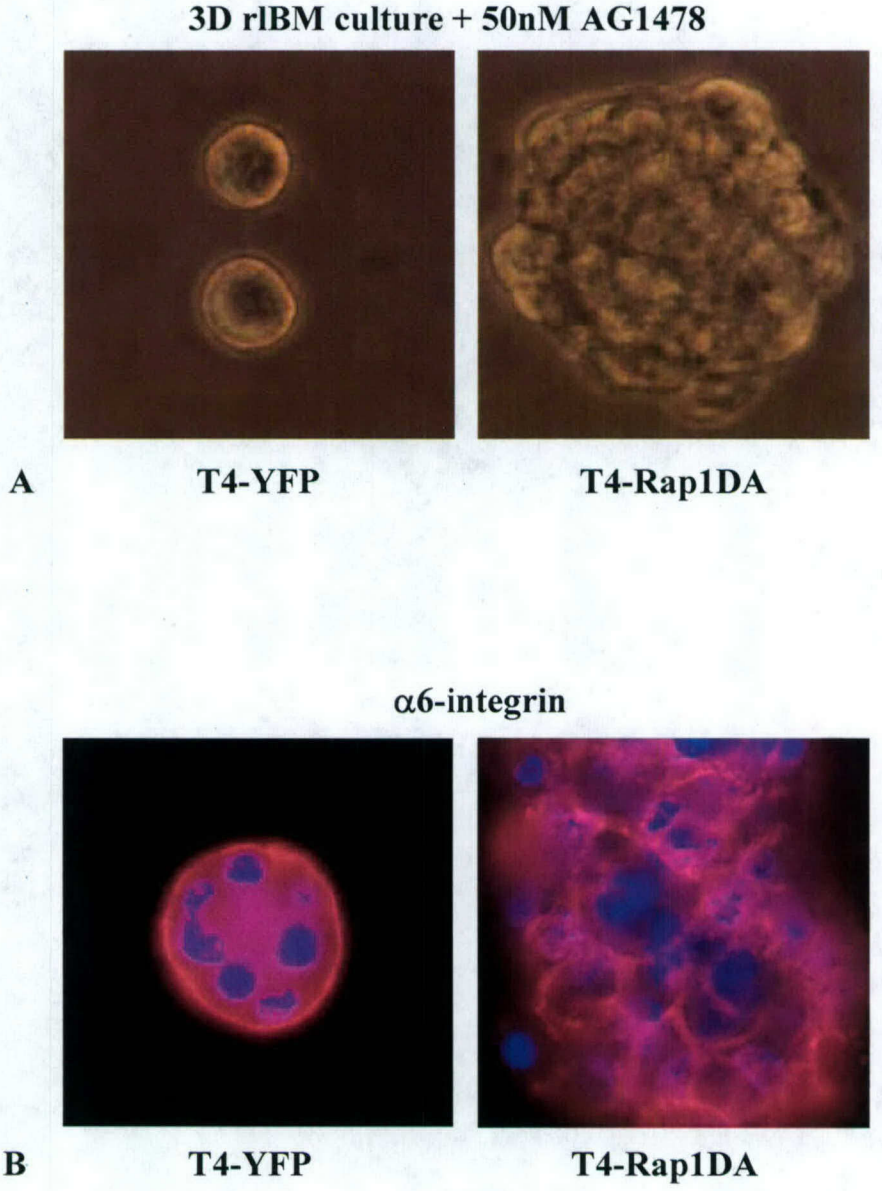


Figure 8 The effect of AG1478 (EGFR inhibitor) on cell growth (**A**) and cell polarity (**B**) in T4-YFP and T4-Rap1DA cells cultured in 3D lrBM.

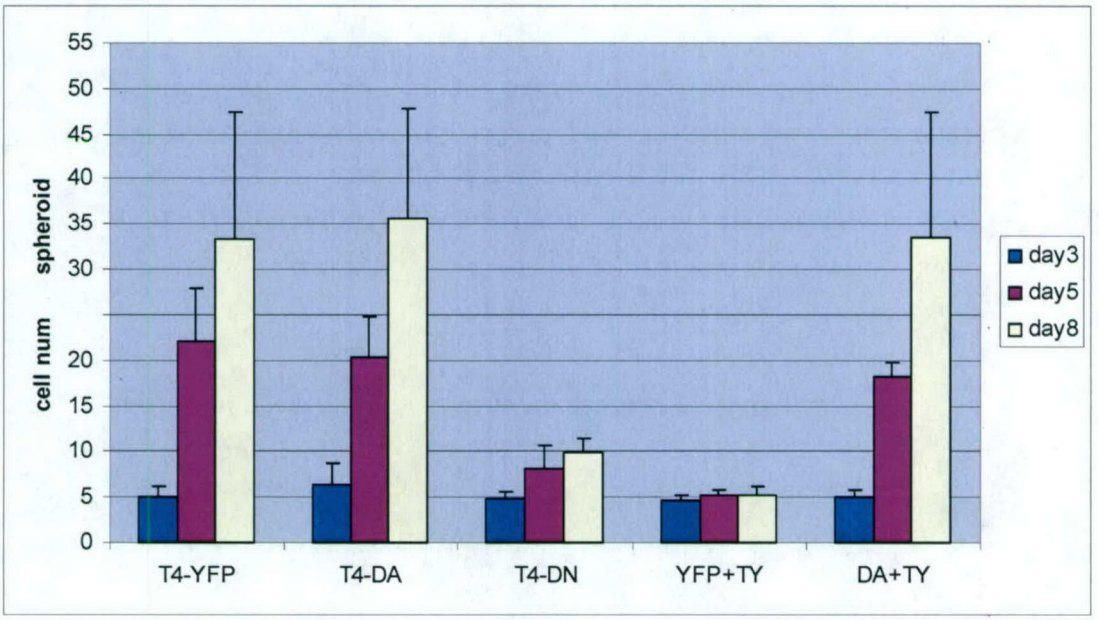
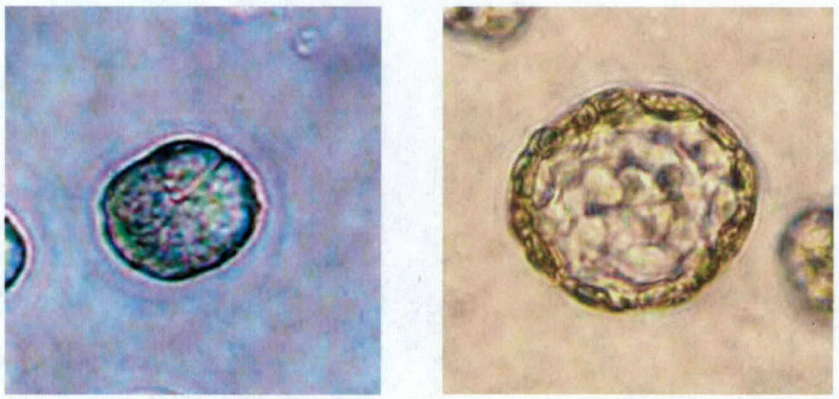


Figure 9 Growth rates of T4-YFP, T4-Rap1DA, and T4-Rap1DN cells cultured in 3D lrBM.

15day Culture



T4-YFP
3D lrBM+AG1478

T4-Rap1DN
3D lrBM

Figure 10 A lumen formation in T4-Rap1DN cells without AG1478.

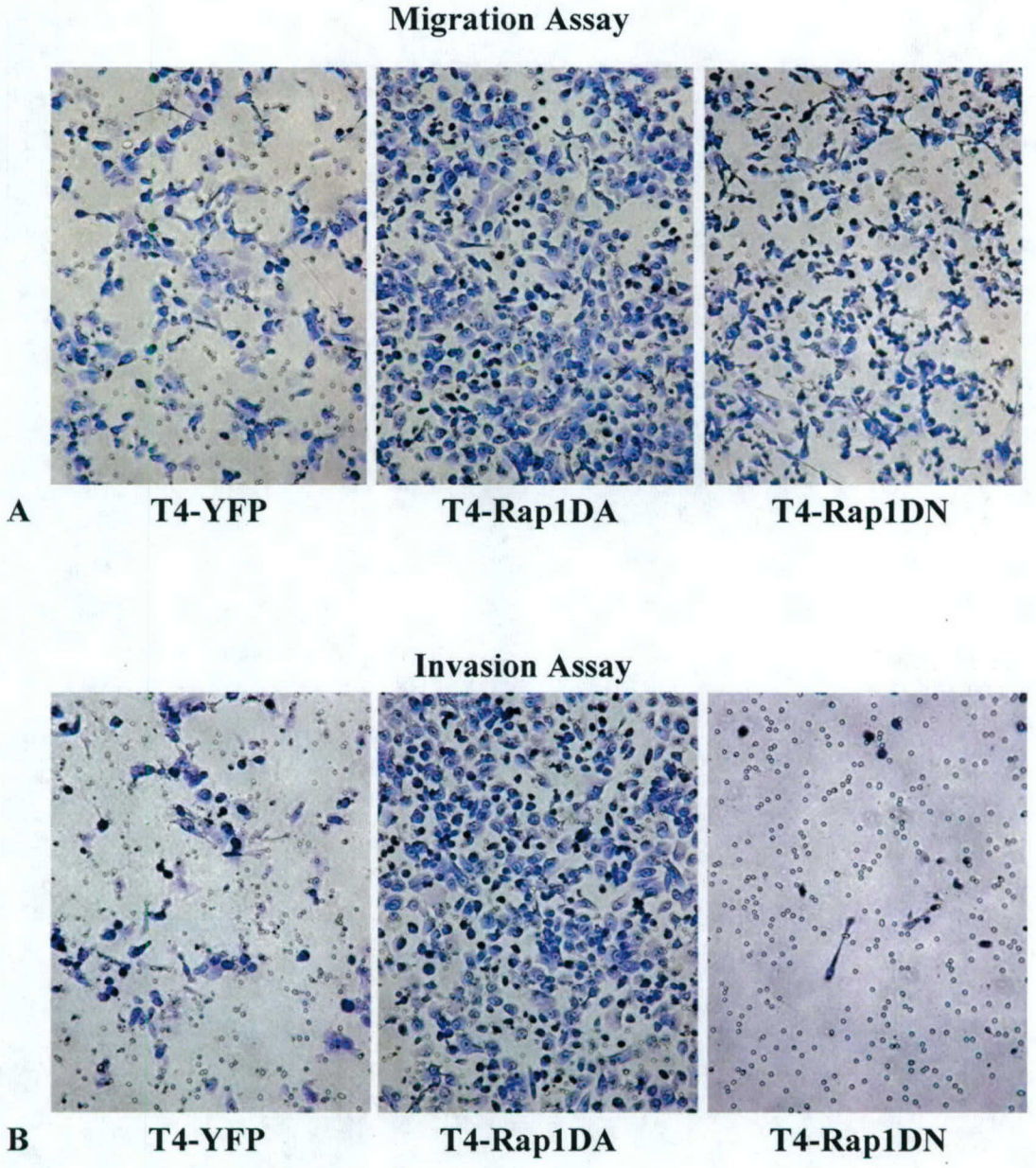


Figure 11 Migration (**A**) and invasion (**B**) of T4-YFP, T4-Rap1DA, and T4-Rap1DN cells with traswell filter assays.

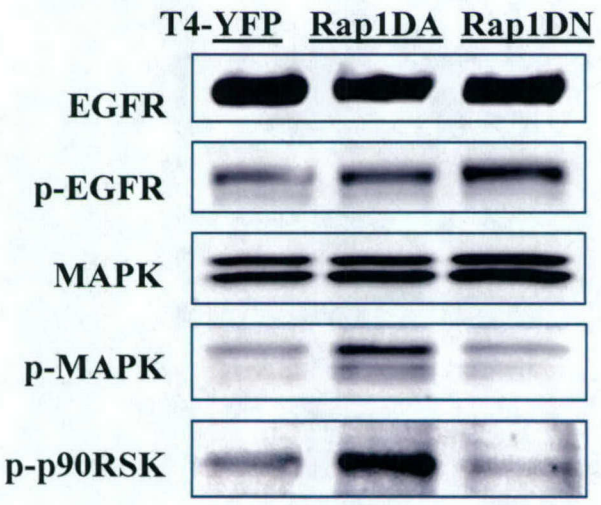


Figure 12 Analyses of EGFR-MAPK pathway in T4-YFP, T4-Rap1DA, and T4-Rap1DN cells.

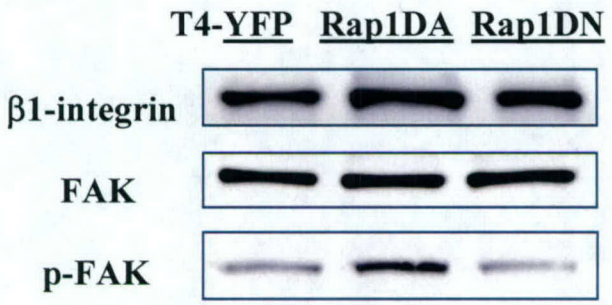


Figure 13 Analyses of integrin pathway in T4-YFP, T4-Rap1DA, and T4-Rap1DN cells.